Metallothionein Induction as a Measure of Response to Metal Exposure in Aquatic Animals

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Metallothioneins (MTs) are considered central in the intracellular regulation of metals such as copper, zinc, and cadmium. Increased MT synthesis is associated with increased capacity for binding these metals and protection against metal toxicity. Recent advances in the biochemistry and molecular biology of MTs have facilitated research on MTs in aquatic species. For the bivalve mollusc *Crassostrea virginica*, a species frequently used in studies on the toxicology and environmental monitoring of metals, the primary structure for MT has been deduced from analysis of the proteins and cDNA. Procedures for analysis of MT synthesis and MT gene expression have been applied in studies of response to metal exposure. Induction of specific MT forms by Cd is concentration- and time-dependent. The levels of MT-bound metals exhibit a strong relationship with the cytosolic metal concentrations in a metal-exposed natural population of oysters. Ribonuclease protection assays using sequence-specific antisense RNA probes have shown that the MT mRNA structure in this natural population exhibits considerable individual variability in the 3′-untranslated region. Although yet to be substantiated, the possibility exists that the distribution of this variability may be related to the level of environmental metal contamination. One probe derived from the coding region is suitable for use in quantitative RPAs for oyster MT mRNAs. — Environ Health Perspect 102(Suppl 12): 91–96 (1994)

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Metallothionein and Toxicology of Metals

The potential importance of metallothionein (MT) in toxicologic responses to metals was recognized at the time of its initial discovery (*1*). These low molecular weight, metal-binding proteins and polypeptides (*2*) are inducible by metals and are believed to participate in functions associated with the metabolism and detoxification of metals (*3*). Although the precise cellular function of MT has remained elusive to investigators, there is considerable evidence to support a purported role in regulating or controlling the intracellular availability of essential metals such as Cu and Zn and the nonessential metal Cd. MTs are capable of donating Cu and Zn to appropriate receptor molecules such as metalloenzymes (*4–6*) and transcriptional factors (*7,8*), thus regulating metal-dependent activities through highly specific molecular interactions. With both essential and nonessential metals, binding to MT limits metal availability at inappropriate sites and is thereby believed to confer protection against toxicity. For proteins previously compromised by binding a toxic metal such as Cd, a rescue function, whereby ZnMT serves as a receptor of Cd and, in the case of Zn metalloproteins, donor of zinc, has been proposed as a mechanism for restoring functional properties of these structures (*9*). Processes that result in increased capacity for MT synthesis, e.g., induction (*10–12*), gene amplification (*13–15*), and gene duplication (*16*), confer cells or individuals with an increased resistance to metal toxicity. In yeast, the ability to resist Cu toxicity is lost when the endogenous MT gene is deleted and restored when a mammalian homologue is inserted in its place (*17,18*). The existence of specific metal-activated transcription factors for MT gene expression serves as evidence that MT induction by metals is a specific cellular response to changes in cellular metal concentrations (*19*).

The ubiquitous distribution of MT in virtually all types of organisms studied to date (*3,20,21*) attests to the conserved nature of MT and its function. Amino acid sequences of varied species including aquatic animals show regions of high similarity (*22*). Conserved nucleotide sequences exist for both coding region and regulatory elements of MTs of mammals, fish, and invertebrates (*23–25*).

The initial reports describing the occurrence of MTs in aquatic animals (*26,27*) were soon followed by proposals for use of MTs in assessing the environmental toxicity of metals (*28,29*). These early proposals were considered controversial due to a lack of general understanding of MT function in aquatic animals. While it is has been known for some time that metals such as Cd, Zn, and Cu can induce MTs in aquatic animals, detailed studies at the cellular and molecular level have only recently been reported (*21*). Application of improved biochemical procedures and recombinant DNA technology has facilitated the analysis of MTs and MT gene expression in several aquatic species. Isolation procedures based on FPLC with fish (*30*) and HPLC with invertebrates (*31,32*) can resolve the multiple forms of MTs in an individual sample. These procedures are necessary for both detailed biochemical analysis and analysis of response to metals. Studies employing molecular approaches are currently advancing our understanding of the structure of the MT DNA and its expression (*22,25,33–43*). The findings should contribute to an increased understanding of MT function in aquatic animals and facilitate evaluation

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of MTs as a possible diagnostic indicator of exposure to metals (*44*).

According to a cellular model for MT induction derived from our current understanding of the regulation of MT gene expression (*19*) and adapted for aquatic species (Figure 1), induction can be assessed at both transcriptional and translational levels. Information associated with an increase in the cellular content of metals is conveyed to the MT gene via metal-activated transcriptional factors that initiate expression following their binding to specific metals. This results in a cascade of events associated with transcription, synthesis of apothionein, and binding of the latter to metal ions to form MT. MT induction can be measured as the concentration or rates of formation of the responsible mRNA, MT, and levels of MT-bound metals. Each of the processes provides different information on the inductive process and may display differential dynamics.

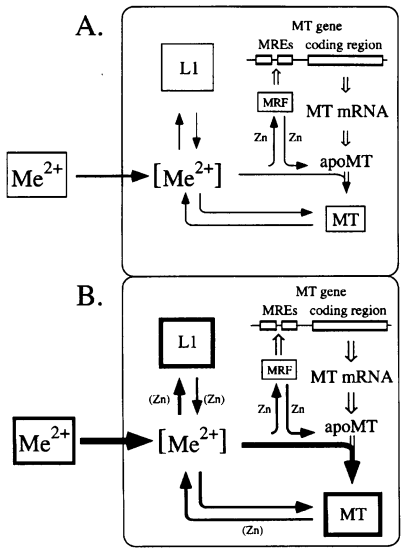

Figure 1. Pathways for intracellular metal distribution and relationship to MT induction. In the uninduced state (*A*), MT synthesis is low and involved in basal metabolism of metals. Metal-dependent transcription is mediated through interactions of metal-regulatory factors (MRF) and the appropriate regulatory elements (MREs). In higher animals, a constitutively active MRF is reported to be under inhibition by a transcriptional inhibitor that can only be released by Zn (*57*). Other metals would induce MT by displacing Zn from intracellular binding sites in L1, making additional Zn available for interacting with the inhibitor. Increased metal influx (*B*) would result in induction of thionein, mediated by displaced Zn, and increased metal flux to MTs. Direct binding of metals to MTs spares other structures from th effects of metals. Additionally, MTs may be involved in metal–metal exchange reactions that result in donation of Zn and sequestration of toxic metals initially bound to target structures in the nonthionein pool L1, thereby effecting repair or rescue of the latter (*9*). In aquatic animals, sequestration of metals can also occur in other cellular structures in L1, such as granules and concretions localized in membrane-bound vesicles. Zn in parentheses denotes that Zn is expected to follow these pathways together with other metals of interest.

Metallothionein Induction in a Mollusc

In aquatic animals, and especially with the invertebrates, the development of procedures for the study of specific MT forms and MT gene expression has been relatively recent. There is still a need to better understand the basic features associated with MT expression and their relationship to cellular mechanisms of metal sequestration and toxicity. Bivalve molluscs such as mussels and oysters are commonly used in environmental monitoring programs because of their ability to concentrate relatively high concentrations of anthropogenically-derived chemicals. Of the various molluscan species, MT induction has been studied in greatest detail in the oyster *Crassostrea virginica*, the first invertebrate species reported to possess MTs (*27*). Cd, Cu, and Zn are known to bind to these proteins (*45,46*). Analysis of the *in vivo* kinetics of Cd-binding by these proteins indicates that they can bind up to 50% of the cellular Cd and that induction results in binding of both newly taken-up Cd ions and redistribution of Cd from other cellular structures to MT (*47*).

The biological half-life of Cd bound to induced MT was estimated as 70 days (*47*) and the half-life of MT as either 4 or 20 days depending on whether turnover measurements were made during or immediately following cessation of exposure (*48*). This discrepancy in half-lives between the bound metal and MT is consistent with an earlier proposal that the metals released during the degradation of MT are rebound to newly synthesized MT, thus extending the turnover time of the metal in relation to protein (*49*). Metals redistributed to MTs from other structures (*47,48*) can also extend the turnover time of MT-bound metals.

The most recent developments in the study of the oyster MTs have been facilitated by application of HPLC isolation procedures (*50*) and molecular probes derived from the MT cDNA (*22*). HPLC coupled with atomic absorption spectrophotometry and polyacrylamide gel electrophoresis have been used to quantify MT or the bound metals, and cDNA probes and antisense RNA probes have been used in Northern blot, dot blot, and ribonuclease protection assage (RPAs) of the mRNA.

The structure of the oyster MT was deduced by N-terminal amino acid sequencing and tandem mass spectrometry of purified proteins (*22,32,48*) and RT-PCR-based cloning and cDNA sequencing (*22*). These studies indicated the presence of two MTs whose sole difference was the absence of an *N*-acetyl group in one. Based on our current understanding of *N*-acetylation of proteins, the oyster MT is expected to exist in the N-blocked form due to the presence of a penultimate serine residue encoded in the mRNA (*48*). The original N-terminal methionine is expected to be removed from the nascent polypeptide during cotranslational processing. This is followed by *N*-acetylation of the new N-terminal serine. It was originally speculated that the appearance of the unblocked form was a maladaptive response due to the effects of Cd on the *N*-acetylation process during the induction of MT (*48*). This view is supported by recent findings that have shown that the unblocked form occurs in high amounts only at relatively high exposure concentrations of 4.4×10^{-1} µM Cd (Figure 2) and higher (*32*). It also appears later than the acetylated form during Cd exposure at these concentrations (*48*). Furthermore, the nonacetylated MT is present in minor amounts, in comparison wth the acetylated form, in a natural population of oysters, which inhabits a metal-contaminated environment (*51*). It appears that MT induction by high concentrations of Cd may create a demand for *N*-acetylation that is not met by the prevailing cellular conditions and is so far seen in significant amounts only in the laboratory. At the present time, there appear to be no differences in functional correlates associated with these forms; i.e., in Cd, Zn, and Cu composition (*32*) or in MT turnover rates (*48*). Nevertheless, it is interesting to speculate whether the appearance of the nonacetylated form is a consequence of the toxicity of Cd.

Induction of MTs (*51*) and the MT mRNA (Unger and Roesijadi, unpublished data) exhibits similar concentration-response relationships at Cd exposure concentrations ranging from about 3.6×10^{-4} µM the background concentration in controls, to 4.4×10^{-1} µM, the highest exposure concentration tested (*51*). An increase over basal levels is observed in both MT and MT mRNA at Cd concentrations greater than 1.0×10^{-1} µM. However, the time courses for the appearance of the two differ

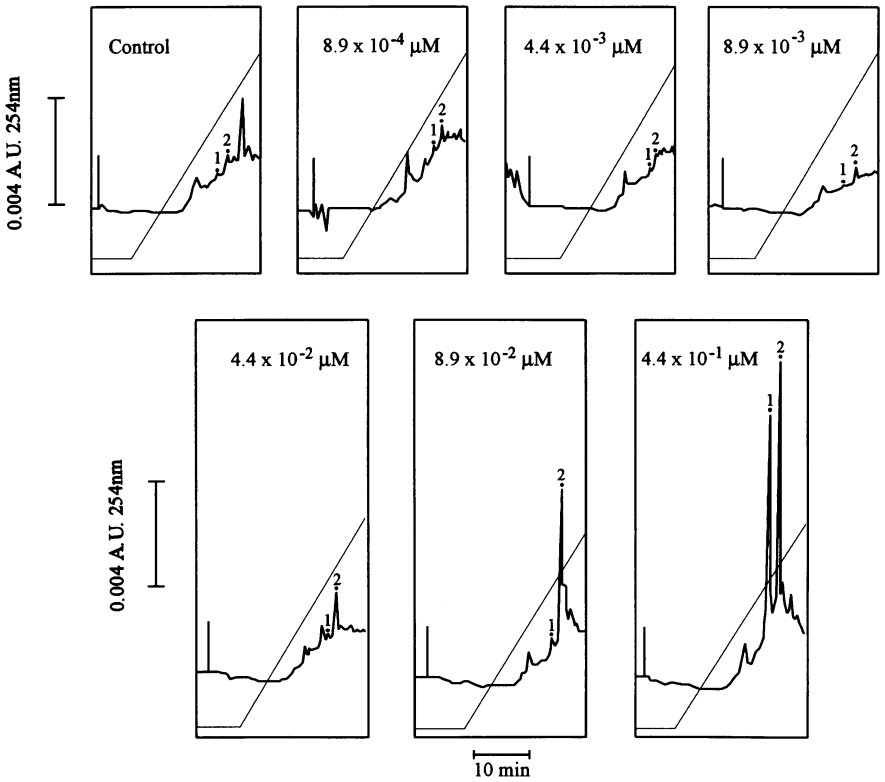

Figure 2. Ion exchange chromatograms (DEAE 5 PW column, TosoHaas) showing concentration–response relationship for induction of gill MTs of *Crassostrea virginica* following exposure of oysters to a range of Cd concentrations for 2 weeks (*51*). A detectable increase in peak 2, the acetylated form of oyster MT (CvNAcMT), is first seen at 4.4 x 10^{-2} µM Cd and continues to increase to 4.4 x 10^{-1} µM Cd the highest concentration shown. Peak 1, the nonacetylated form (CvMT), is detected only at the highest concentration.

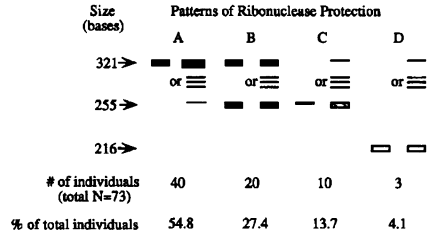

Figure 3. Individual variability in MT RNA structure in a natural population of oysters (*55*). Ribonuclease protection assays were conducted on total RNA of individual oysters hybridized to radiolabeled antisense RNA probes encoding most of the coding region and the entire 3′ UTR. Representative profiles of ribonuclease-protected oyster MT RNAs for individual oysters in a natural population of oysters are shown. The variability observed among individuals, shown in lanes A through D, was localized to the 3′ UTR of the oyster MT RNA using a battery of RNA probes that were antisense to either the coding region, the UTR, or both.

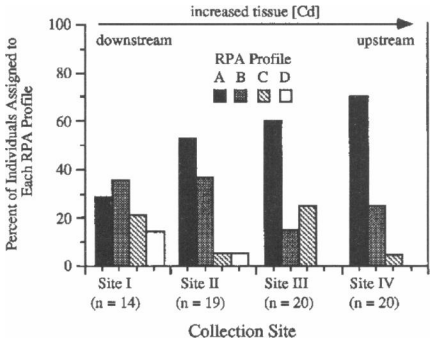

Figure 4. Distribution of individual profiles for ribonuclease protection of oyster MT mRNA from four locations on the Patuxent River, a metal-contaminated environment (*55*). Profiles for individual oysters were assigned to one of the four patterns shown in Figure 3, lanes A through D. The distribution of the different profiles of RNase protection in oysters collected from different locations on the river suggests a relationship between the occurrence of the different types of RNAs and a contamination gradient in tissue Cd concentrations going upstream from Station I to Station IV.

during induction by Cd. Increases in MT levels lag considerably in comparison with MT mRNA levels. At 4.4 × 10^{-1} µM Cd, the increase in mRNA levels appears hyperbolic and approaches a maximum after 1 day of exposure. At this same exposure concentration, MT levels can continue to increase for durations up to 24 days (*47*). However, rates of MT synthesis determined by ^{35}S-cysteine pulse labeling indicate the establishment of new rates by 7 days (*48*).

Metallothionein in a Natural Population

In parallel with the laboratory studies, the behavior of MTs has been examined in a natural population of oysters in the Patuxent River, Maryland, a metal-contaminated tributary of the Chesapeake Bay. The existence of high concentrations of metals such as Ag and Cu in oysters in this estuarine system is well documented (*52,53*). We have also reported that similar elevations exist with Cu, Cd, and Zn in gill tissues, our standard tissue for study of MT function (*51*). Tissue Cd concentrations exhibit a linear relationship with an apparent contamination gradient in this estuarine system (*51*). When levels of gill Cd, Cu, and Zn bound to MTs were compared with the total accumulated concentrations of each of the metals, MTs had the greatest impact on the accumulated Cd levels; on average, accounting for 21.6% of the total tissue Cd in comparison with 0.3% and 0.9% for Zn and Cu, respectively. However, the relationship between MT-bound and total accumulated Cd was not linear, and the direct correspondence was observed with MT-bound Cd and Cd accumulated in the cytosol. Oysters possess other mechanisms for metal sequestration (*54*), and these appear to play a significant role in binding Cd, Cu, and Zn in oysters in the Patuxent River. Their relative importance also appears to vary, thus accounting for the variance between MT-bound and total accumulated metal concentrations.

When the MT mRNA in this population of oysters was examined using RPAs (*55*) individual variability in the sizes of RNase-protected fragments suggested individual variability in the MT mRNA structure (Figures 3,4). The patterns of individual variability could be assigned to one of four groups. These included one with the full-length protected fragment as the main protected band and three others with smaller protected fragments as the main bands. In two of the groups, the full-length protected band was completely absent. The probe that resulted in these initial observations was antisense RNA containing sequence for both the coding region and the 3′-untranlated region (3′-UTR) of oyster MT. This probe was derived from a cDNA clone isolated from a selected, cultured strain of oysters exposed to Cd (*22*).

It was possible to further localize the region of variability in the MT mRNAs in the natural population by using shorter probes antisense to either the 3′-UTR or the coding region. RNase protection with the 3′-UTR probe resulted in the same type of variability in the pattern of protected fragments as seen with the original probe; while use of the coding region probe resulted in a single band of the expected size for a fully-protected fragment. The structure of the coding region of the MT mRNAs is, therefore, similar among individuals and expected to encode the same protein. The individual variability exists in the 3′-UTR. The 3′-UTR is recognized as having roles in determining RNA localization, polyadenylation, stability, and translation initiation (*56*), and variability in this region of the MT mRNA may signify differences in the utilization or regulation of MTs by individuals of differing backgrounds. Preliminary evidence (Figure 4) is suggestive of the possibility that the variability may be related to a Cd contamination gradient in oyster tissues. However, additional data are required for definitive conclusions. Either alternative splicing of similar primary transcripts or genetic polymorphism will most likely represent the underlying mechanism responsible for the observed variability. The functional significance of the variability remains to be determined.

Although the variability described above precluded using the "full-length" antisense probe in quantitative RPAs for total MT mRNAs, the probe derived from the coding region is suitable in such assays and is currently being used in a protocol based on the standard additions method. A sense-strand transcript derived from the same template as the probe is used as the standard.

Summary

The preceding account has summarized the basis for investigating MTs in the context of the environmental toxicology of metals and recent progress on understanding the response of MTs of an estuarine mollusc as a function of metal exposure. Continued application of biochemical and molecular approaches to the study of MTs of aquatic organisms should facilitate investigations on functional aspects of MT induction and the behavior of MTs in natural populations.

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